Atovaquone Maintenance Therapy Prevents Reactivation of Toxoplasmic Encephalitis in a Murine Model of Reactivated Toxoplasmosis

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Acute therapy with pyrimethamine plus sulfadiazine is the treatment of choice for reactivated toxoplasmic encephalitis (TE). Acute therapy is followed by lifelong maintenance therapy (secondary prophylaxis) with the same drugs at lower dosages. The use of pyrimethamine plus sulfadiazine is hampered by severe side effects including allergic reactions and hematotoxicity. Alternative treatment regimens with pyrimethamine plus clindamycin or other antiparasitic drugs are less efficacious. Atovaquone nanosuspensions show excellent therapeutic effects for "acute" intravenous (i.v.) treatment of reactivated TE in a murine model. In the present study, the therapeutic efficacy of atovaquone for oral "maintenance" therapy was investigated. Mice with a targeted mutation in the interferon regulatory factor 8 gene were latently infected with *Toxoplasma gondii*, developed reactivated TE, and received acute i.v. therapy with atovaquone nanosuspensions. Mice were then treated orally with atovaquone suspension or other antiparasitic drugs to prevent relapse of TE. Maintenance therapy with atovaquone at daily doses of 50 or 100 mg/kg (body weight) protected mice against reactivated TE and death. This maintenance treatment was superior to standard therapy with pyrimethamine plus sulfadiazine. The latter combination was superior to the combination of pyrimethamine plus clindamycin. Inflammatory changes in the brain parenchyma and meninges, as well as parasite numbers, in the brains of mice confirmed the therapeutic efficacy of atovaquone for maintenance therapy. Atovaquone was detectable in sera, brains, livers, and lungs of infected mice by high-performance liquid chromatography and/or mass spectrometry. In conclusion, atovaquone appears to be superior to the standard maintenance therapy regimens in a murine model of reactivated TE. The therapeutic efficacy of atovaquone for maintenance therapy against TE should be further investigated in clinical trials.

Toxoplasma gondii is an intracellular protozoan parasite of humans and animals with worldwide distribution. Seroprevalence varies with geographical location and reaches 70% in Germany and France (7, 21). After initial uptake of the parasite in the gut and dissemination throughout the body, the latent stage of infection is characterized by the presence of parasites in cysts in the central nervous system and muscle tissues (21). Immunocompromised hosts, i.e., patients with AIDS or organ transplant recipients, are at risk of reactivation of the infection by rupture of cysts (21). Toxoplasmic encephalitis (TE) is the most common clinical manifestation of reactivated disease in AIDS patients who do not receive highly active antiretroviral therapy (HAART) or antiparasitic prophylaxis. TE is the most frequent infectious cause of focal intracerebral lesions in these patients (20–22). Untreated, reactivation of disease leads to death of the patient. The acute therapy (pyrimethamine plus sulfadiazine) of TE is followed by lifelong maintenance therapy (19, 21). The standard regimen for maintenance therapy includes pyrimethamine plus sulfadiazine at lower dosages (21). Pyrimethamine plus sulfadiazine therapy is hampered by severe side effects, including hematologic toxicity and/or life-threatening allergic reactions in 5 to 15% of patients (16, 19).

The hydroxynaphthoquinone atovaquone is a potent inhibitor of the respiratory chain of parasites with potent in vitro and in vivo activity against both the tachyzoite and cyst forms of *T. gondii* (1, 3, 5, 18, 25). The original formulation of atovaquone (750-mg tablets four times daily) as a single antitoxoplasmic agent was reported to prevent relapse in 48 of 65 (76%) AIDS patients with mean CD4 counts of 29/µl (17). More recently, a new formulation of atovaquone (1,500 mg suspension) in combination with pyrimethamine or sulfadiazine was reported to prevent relapse in 19 of 20 (95%) patients (5).

We have previously shown the efficacy of atovaquone nanosuspensions in the "acute" intravenous (i.v.) treatment of TE in a murine model (25). To investigate the therapeutic efficacy

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of atovaquone for oral "maintenance" therapy, we expanded the murine model of reactivated TE in mice deficient in the interferon regulatory factor 8 (ICSBP/IRF-8) (14) by adding a phase of oral "maintenance" treatment after the course of acute i.v. drug.

Results of the present study reveal that atovaquone maintenance therapy in doses equivalent to the application in humans protected mice against reactivated TE and death. Atovaquone-treated mice did not develop signs of inflammation in the brain parenchyma or in the meninges. Atovaquone maintenance therapy was superior to standard therapy with pyrimethamine plus sulfadiazine for secondary prophylaxis of TE.

MATERIALS AND METHODS

T. gondii. Cysts of the ME49 strain of *T. gondii* were obtained from brains of NMRI mice that had been infected intraperitoneally with 10 cysts 2 to 4 months before. Mice were sacrificed by asphyxiation with CO_2, and their brains were removed and triturated in phosphate-buffered saline (PBS). An aliquot of the brain suspension was used to determine the numbers of cysts in the preparation by microscopy.

Mice and infection. Inbred female ICSBP/IRF-8$^{-/-}$ mice on a C57BL/6 background were bred and maintained under specific-pathogen-free conditions in the animal facility of the Institute for Infection Medicine, Charité Campus Benjamin Franklin, Berlin, Germany. Eight- to twelve-week-old ICSBP/IRF-8$^{-/-}$ mice were orally infected with 10 cysts. Mice were treated with sulfadiazine (Sigma-Aldrich, Deisenhofen, Germany) in drinking water (400 mg/liter) for 4 weeks beginning 2 days after infection to control latent infection. Two days after discontinuation of sulfadiazine, mice were treated with atovaquone nanosuspensions (10.0 mg/kg [body weight]) administered as a single i.v. dose on days 2, 5, and 8. At day 9 after discontinuation of sulfadiazine (1 day after discontinuation of acute i.v. atovaquone therapy), daily treatment with different antiparasitic drugs as maintenance therapy was initiated perorally for 1 week. At day 16 (the time point at which control mice showed symptoms of disease and/or began to succumb), the brains, livers, lungs, and sera were removed and fixed in formalin for histology or stored at −70°C for high-performance liquid chromatography (HPLC) and mass spectrometry (MS) analysis. There were four to six mice in each experimental group to study mortality and histological changes. HPLC and MS analysis were performed on organs and serum samples of three to four mice per group. Experiments were repeated at least three times.

Atovaquone. Atovaquone (2-[trans-4-(4-chlorophenyl)cyclohexyl]-3-hydroxy-1,4-naphthoquinone) was obtained from Glaxo-SmithKline (Munich, Germany). Atovaquone nanosuspensions for acute i.v. therapy were produced by high-pressure homogenization under aseptic conditions. The drug powder (Glaxo-SmithKline) was dispersed in an aqueous surfactant solution containing 1% Tween 80 (ICI Surfactants, Eversberg, Belgium) by using Ultra Turrax T25 (Janke and Kunkel, Staufen, Germany). The coarse predispersion obtained was homogenized in a Micron LAB 40 high-pressure homogenizer (APV Systems, Unna, Germany) applying pressures of 150 and 500 bar (2 cycles each) and 1,500 bar (20 cycles) (11). Particles were preserved by thiomersal (Sigma-Aldrich) at a concentration of 0.001% (wt/vol). Iso-osmolarity was achieved by adjusting with glycerol (Sigma-Aldrich) at a concentration of 2.25% (wt/vol). Particle size and width of distribution (polydispersity index) were determined by photon correlation spectroscopy (Malvern Zetasizer 4; Malvern Instruments, Malvern, United Kingdom) and laser diffractometry with a Coulter LS 230 (Beckman-Coulter, Krefeld, Germany). The mean diameter measured by photon correlation spectroscopy was 459 ± 2 nm, with a polydispersity index of 0.29 ± 0.02; 99% (vol/vol) of the particles had a diameter of <2.155 μm (LD99). Atovaquone suspension (Wellvone; Glaxo-SmithKline) for oral maintenance therapy was diluted in PBS. Mice received 100, 50, or 25 mg/kg (body weight) in a total volume of 0.2 ml by gavage.

Histology. Organs were excised, fixed in a solution containing 5% formalin, and embedded in paraffin. Sagittal sections of brains and cross sections of livers and lungs were stained with hematoxylin and eosin (H&E) according to standard procedures or by an immunoperoxidase method with rabbit anti-*T. gondii* immunoglobulin G antibody (6). All reagents for fixing and staining with H&E were obtained from Merck (Darmstadt, Germany). For staining by the immunoperoxidase method, deparaffinized sections were incubated with swine sera at 1:10 (Dako, Carpinteria, Calif.) and with the primary antibody rabbit anti-*T. gondii*. For production of rabbit anti-*T. gondii* antibodies, rabbits were orally infected

with 10 cysts of strain ME49 and treated with 300 mg of sulfadiazine/liter. Sera were harvested after a boost with *T. gondii* (RH strain) 15 days after infection. After a rinse with modified PBS, sections were incubated with swine anti-rabbit immunoglobulin (1:100; Dako). Finally, sections were incubated with rabbit peroxidase anti-peroxidase (1:100; Sigma-Aldrich) and with diaminobenzidine (Dako) development solution after a rinsing step. Sections stained with H&E were evaluated for inflammatory changes, and sections stained by the immunoperoxidase method were evaluated for the numbers of *T. gondii* cysts and *T. gondii* tachyzoites or antigens. The numbers of inflammatory foci and parasites were counted under ×100 magnification in three optical fields. A total of three sections of each organ from four mice in each experimental group were evaluated for the numbers of inflammatory foci and the numbers of *T. gondii* cysts, tachyzoites, and antigens. A modification of the score described by Araujo et al. was used (2). Briefly, normal brain was scored 1. A score of 2 reflected mild meningeal and parenchymal mononuclear cell inflammation, a score of 3 indicated severe meningeal and parenchymal inflammation, and a score of 4 was used for severe meningeal inflammation plus severe parenchymal inflammation with necrosis. The score is given as mean ± the standard deviation for at least four mice in each group.

HPLC. Weighted samples of serum (200 μl) and liver and lung (50 to 300 mg) were homogenized in 5 ml of extraction solution consisting of 2% (vol/vol) isoamyl alcohol and 98% (vol/vol) hexane in a glass-Teflon homogenizer (12). Then, 0.1 ml of serum was diluted in 5 ml of extraction solution. After the addition of 1 ml of phosphate buffer, samples were spun for 20 min in a rotating mixer (13). Suspensions were centrifuged for 10 min at 2,800 × g in a temperature-controlled centrifuge at 10°C. Then, 4 ml of supernatant was evaporated to dryness in a rotating vacuum centrifuge. The dry residue was redissolved in mobile phase (aqueous solution of 50% [vol/vol] acetonitrile and 5% [vol/vol] methanol; pH 2.65) (8, 12, 13). The samples were chromatographed on a reversed-phase column (Spherisorb C1; Waters) guarded with a C_{18} precolumn in isocratic mode. Absorbance of the eluate was monitored at 254 nm in a UV detector (model LC 95; Perkin-Elmer, Überlingen, Germany). The linear calibration function was calculated by means of least-squares regression analysis by using computer software (SQS 98; Perkin-Elmer). The detection limit of this method was 0.6 mg/liter of serum. Limits of quantitation for tissues were ca. 0.5 mg/kg tissue. Interassay precision for serum varied from 7.4 to 15.1%. Recovery from spiked serum was 98.1 to 108.1%. Replicate extractions yielded the following extraction rates for the first extraction: 100.0% (serum), 63.6% (brain), 78.1% (liver), and 78.1% (lung).

MS. Since atovaquone concentrations were low in the brains of mice undergoing atovaquone maintenance therapy, we used MS to quantitate atovaquone in the brains of these mice. We mixed 25-μl aliquots of serum with 100 μl of internal standard solution containing mycophenolat (Roche, Grenzach, Germany) at a concentration of 0.3 mg/ml. After the addition of 300 μl of acetonitrile (VWR-International, Darmstadt, Germany), samples were vortexed and sonicated for 15 min at room temperature. Samples were then centrifuged at 13,000 × g for 6 min. The supernatants were transferred to clean tubes, and aliquots of 20 μl were injected onto a reversed-phase C_{18} column (Eurospher; 5μ; 4.6 mm by 30 mm; Knauer, Berlin, Germany). Mobile phase A was distilled water containing 0.0025 M ammonium acetate (VWR-International). Mobile phase B was acetonitrile-ammonium hydroxide (100/0.008%). The HPLC system consisted of a Rheos 2000 HPLC pump (Flux Instruments, Basel, Switzerland) and a 233XL Autosampler (Gilson Abimed). HPLC separation was achieved with mobile-phase gradient elution (flow, 1.5 ml/min) by using the following sequence: 0 min at 100% phase A, 0.1 min at 25% phase A, 3.0 min at 25% phase A, and 3.1 min at 100% phase A. The majority (80%) of the effluent was split off before entering the MS.

An API 3000 mass spectrometer (Applied Biosystems) equipped with an electrospray ionization interface and run with Analyst 1.2 software was used for detection and quantification of atovaquone in serum and brain samples. Analytes were monitored in the negative MRM mode with the following transitions of precursor to product ions: *m/z* 365.1 to 171.2 (atovaquone) and *m/z* 318.6 to 275.2 (mycophenolat). The source temperature was set to 400°C.

Standards and quality control samples were prepared in blank mouse serum. For each batch, an eight-point standard calibration curve was analyzed; atovaquone concentrations ranged from 0.617 to 79.0 mg/ml.

For quantification of atovaquone in brain tissue the organs were weighed into polypropylene microreaction vials and homogenized mechanically using a pestle. After the addition of acetonitrile (5 μl/mg brain tissue), the samples were vigorously vortexed and sonicated for 60 min. The suspended brain tissue was sedimented at 13,000 × g for 6 min; 250-μl aliquots of the supernatant (containing the extract of 50 mg of brain tissue) were transferred to clean polypropylene vials, and 20 μl of the internal standard solution (mycophenol at 0.3

TABLE 1. Effect of infection and atovaquone concentrations in organs in *T. gondii*-infected mice dependent on acute i.v. treatment regimen[a]

Drug	Treatment regimen	Dosage (mg/kg)	Mortality[b] (%)	Mean no. of inflammation foci in brain[c] ± SD	Mean no. of parasites in brain ± SD	Atovaquone concn[d] in:		
						Serum (mg/liter)	Brain (mg/kg)	Liver (mg/kg)
Control[e]	NA[f]		100	NP	NP	ND	ND	ND
Atovaquone	Every 2 days	10	0	0	0	NP	NP	NP
	Every 3 days	10	0	0	0	35.1 ± 7.7	1.9 ± 0.5	35.5 ± 12.3
	Every 3 days	5	20*	1.16 ± 0.34†	7.3 ± 3.6†	0.4 ± 0	ND	2.5 ± 1.7
	Every 3 days	2.5	50†	3.2 ± 1.13†	24.5 ± 3.5†	ND	ND	6.9 ± 1.8

[a] *, Not significant compared to control mice; †, $P < 0.02$ compared to control mice; NP, not performed; ND, not detectable.
[b] That is, the total mortality at day 10 after acute therapy with atovaquone nanosuspensions.
[c] That is, the number of inflammatory foci per optical field under magnification at × 100.
[d] Atovaquone concentrations were determined by HPLC.
[e] Since all control mice died before the day of section, histology could not be performed in this group.
[f] NA, not applicable.

mg/ml) was added. Chromatographic and MS conditions were as described above. Six calibration standards prepared from blank mouse brain tissue were analyzed with each run. Standard concentrations ranged from 0.051 to 3.29 mg/kg.

Statistical analysis. The Fisher exact test was used to compare survival rates. Differences in numbers of inflammatory foci and parasite numbers were analyzed by using the Student *t*, alternate Welch *t*, or Wilcoxon rank-sum test.

RESULTS

Determination of optimal time of administration and dosage of atovaquone for acute i.v. treatment of reactivated toxoplasmosis. We have previously reported that mice acutely treated i.v. with atovaquone nanosuspensions (10 mg/kg [body weight]) do not develop reactivated toxoplasmosis, whereas all control mice died within 2 weeks after withdrawal of sulfadiazine (administered to establish latent infection) (25). To determine the maximum duration between i.v. injections of atovaquone nanosuspension, the treatment of mice with 10 mg/kg (body weight) of atovaquone every other day or every third day was compared (Table 1). Both treatment regimens showed equal therapeutic efficacies in the murine model of reactivated toxoplasmosis. While all control mice died, mice treated with 10 mg of atovaquone/kg every second or third day survived the infection (Table 1). Treated mice did not develop parasite-associated inflammatory changes in their brains (Table 1). The optimal dose of i.v. atovaquone for treatment of acute reactivated toxoplasmosis was determined by comparing treatments with 10, 5, and 2.5 mg of atovaquone nanosuspension/kg (body weight). Whereas mice treated with 10 mg of atovaquone nanosuspensions did not develop parasite-associated inflammatory changes and survived the infection, 20 and 50% of mice treated with a dose of either 5 or 2.5 mg of atovaquone nanosuspensions, respectively, developed parasite-associated inflammatory changes in their brains and died. High concentrations of atovaquone were only detectable by HPLC in the sera and organs of mice treated with 10 mg/kg (Table 1). In contrast, mice treated with 5 mg/kg showed low atovaquone concentrations in serum and liver, whereas atovaquone was not detectable by HPLC in brains. Therefore, 10-mg/kg atovaquone nanosuspensions administered every third day were used for i.v. treatment of acute reactivated toxoplasmosis in all experiments to investigate the therapeutic efficacy of a variety of antiparasitic drugs for subsequent maintenance therapy.

Murine model of reactivated TE for the evaluation of maintenance therapy. After modification of the acute treatment phase as described above, we expanded the murine model of acute reactivated toxoplasmosis to include maintenance therapy (Fig. 1). Reactivation of latent toxoplasma infection was induced by the withdrawal of sulfadiazine (used to establish latent infection in immunocompromised mice) (25). Two days later when the acute-infection therapy was reactivated in mice, atovaquone nanosuspensions were initiated every third day at a dose of 10 mg/kg (days 2, 5, and 8 after discontinuation of sulfadiazine). One day later, oral maintenance treatment with different antiparasitic drugs was started and administered daily by gavage for 7 days (Fig. 1). Sixteen days after discontinuation of sulfadiazine, sera and organs were obtained and the mortality of mice was monitored in a separate group of mice.

Effect of atovaquone maintenance therapy on survival of mice with reactivated TE. One day after completion of acute i.v. therapy with atovaquone nanosuspensions, mice were treated with different antiparasitic drugs for 7 days. Control mice began to die within 6 days after discontinuation of acute treatment; all control mice died within 9 days (Fig. 2). In contrast, all mice orally treated with atovaquone suspensions as maintenance therapy (100 mg/kg) survived the infection until the end of the observation period (10 days). The same survival rate was observed in mice treated with 50-mg/kg atovaquone suspension. The combination of pyrimethamine (0.71 mg/kg) plus sulfadiazine (30 mg/kg) administered orally (in doses equivalent to those used in AIDS patients) provided partial protection against reactivation; all mice survived the maintenance treatment period of 7 days. Starting on day 8 after initiation of maintenance treatment, these mice began to die. The mortality was 14.3% at day 10 after initiation of maintenance therapy (Fig. 2). The combination of pyrimethamine (0.71 mg/kg) plus clindamycin (35 mg/kg) showed a trend toward inferior efficacy compared to the treatment with atovaquone ($P = 0.0976$). Mice treated with trovafloxacin started to die at 8 days after initiation of maintenance treatment. By day 10, the mortality was 34.0%. Sulfadiazine when administered in drinking water did not protect mice against reactivation of TE (Fig. 2).

Effect of atovaquone maintenance therapy on histological changes in mice with reactivated TE. Histological findings in brains and livers obtained on day 7 after initiation of maintenance therapy (the time point when maintenance therapy was stopped) paralleled the results of survival described above (Table 2 and Fig. 3). Control mice developed severe meningeal
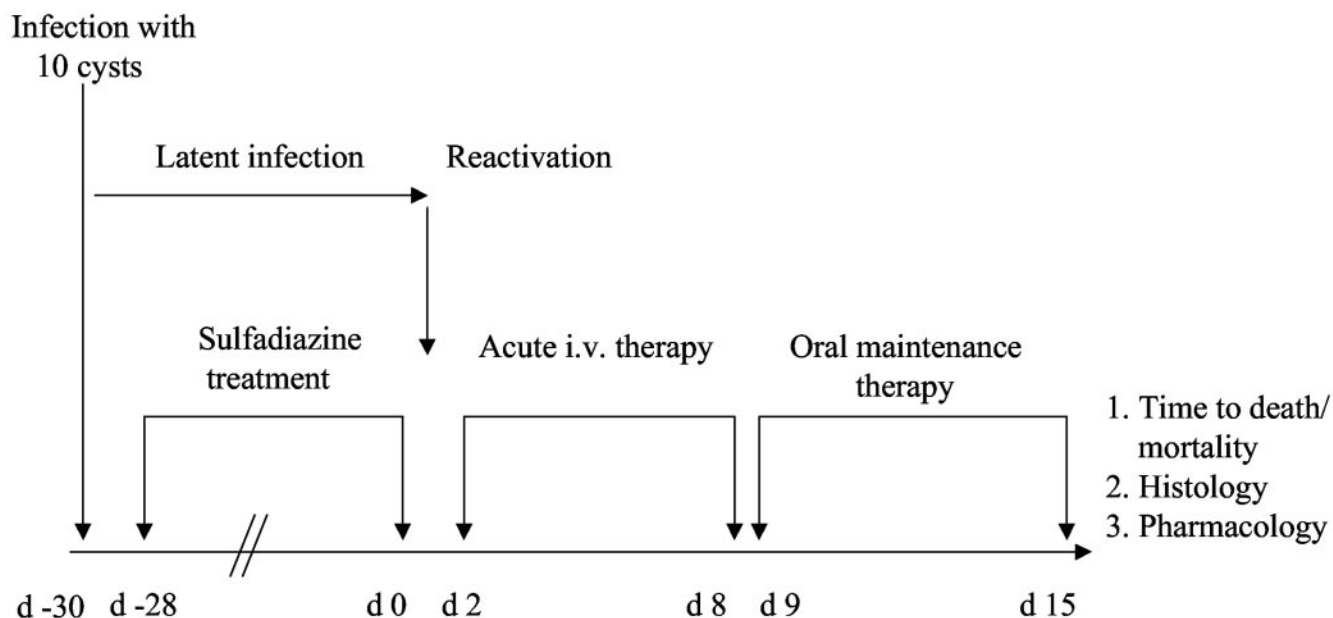
FIG. 1. Murine model of reactivated toxoplasmosis in ICSBP/IRF-8⁻/⁻ mice. After reactivation of latent toxoplasmosis, acute i.v. therapy with atovaquone nanosuspensions was given for 6 days, followed by oral maintenance therapy administered for 7 days (see Materials and Methods). d, day(s).

and parenchymal inflammation with numerous parasites and parasite antigens (Fig. 3A); cysts were also present in low numbers (data not shown). In the livers of control mice, we detected numerous areas of inflammation associated with parasites (Table 2). In contrast, atovaquone maintenance therapy prevented the development of TE; neither mice treated at 100 mg/kg nor those treated at 50 mg/kg showed any signs of inflammation in their brains (Fig. 3B and C) or livers. Parasites were not detectable in either organ. Similar results were obtained in mice treated with pyrimethamine plus sulfadiazine (Fig. 3D). However, signs of inflammation were observed. In contrast, the brains of mice treated with pyrimethamine plus clindamycin showed moderate meningeal and parenchymal inflammation (Fig. 3E); low numbers of parasites were detectable primarily in areas of inflammation. The numbers of inflammatory foci in the meninges and the brain parenchymas were similar in mice treated with trovafloxacin and in control mice; however, parasite numbers were significantly lower in the brains of trovafloxacin-treated mice compared to control mice (Table 2 and Fig. 3F).

Atovaquone concentrations in serum and organs. To determine atovaquone concentrations, sera, brains, lungs, and livers were obtained on day 8 after the initiation of maintenance therapy. Atovaquone concentrations in serum samples were determined by HPLC and MS 24 h after the last administration of drug; mice orally treated with 50 or 100 mg of atovaquone/kg showed high drug concentrations of 15.00 mg/liter or higher in their sera (Fig. 4A). Atovaquone was detectable in livers and lungs at lower concentrations (Fig. 4B). The mean atovaquone concentrations in the brains of mice treated with either 50 or 100 mg/kg were 0.22 ± 0.05 or 0.34 ± 0.14 mg/kg, respectively (Fig. 4B).
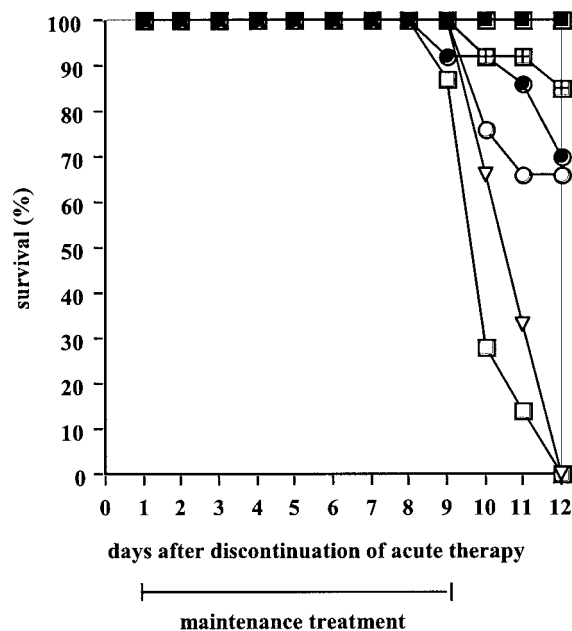
FIG. 2. Survival rate of ICSBP/IRF-8⁻/⁻ mice treated with atovaquone (50 or 100 mg/kg [body weight]) or control drugs. After reactivation of latent toxoplasmosis, acute i.v. therapy with atovaquone nanosuspensions was given for 6 days, followed by oral maintenance therapy administered for 7 days (see Materials and Methods). At least five mice were used in each group. The results shown are pooled from two independent experiments. Symbols: ■, atovaquone (100 and 50 g/kg); ⊞, pyrimethamine (0.71 mg/kg) plus sulfadiazine (30 mg/kg); ●, pyrimethamine (0.71 mg/kg) plus clindamycin (35 mg/kg); ○, trovafloxacin (200 mg/kg); ▽, sulfadiazine (400 mg/liter in drinking water); □, control.

TABLE 2. Histologic findings in brains and livers of mice under maintenance treatment for the prevention of TE[a]

Treatment regimen	Mean no. ± SD[b] in:				
	Brain			Liver	
	Inflammatory foci in parenchyma	Meningeal inflammation	Parasites	Inflammatory foci	Parasites
Control	4.78 ± 0.97	2.83 ± 0.75	34.9 ± 26.37	15.27 ± 7.73	3.11 ± 3.06
Atovaquone					
100 mg/kg	0 ± 0*	0.0 ± 0*	0 ± 0*	0 ± 0*	0 ± 0*
50 mg/kg	0 ± 0*	0.0 ± 0*	0 ± 0*	0 ± 0*	0 ± 0*
Pyrimethamine + sulfadiazine	0 ± 0*	0.0 ± 0*	0 ± 0*	2.0 ± 1.05*	0 ± 0*
Pyrimethamine + clindamycin	1.56 ± 1.13‡	1.02 ± 0.44‡	2.33 ± 3.57*	4.67 ± 1.87*	2.0 ± 2.08‡
Trovafloxacin	1.73 ± 1.42‡	1.43 ± 0.53‡	5.6 ± 4.09*	0 ± 0*	0 ± 0*
Sulfadiazine	4.5 ± 1.09‡	2.83 ± 0.40‡	30.0 ± 22.68‡	4.0 ± 2.41†	2.14 ± 2.04‡

[a] Reactivation of TE was induced in IRF-8^{−/−} mice infected with 10 cysts of the ME49 strain of *T. gondii* by withdrawal of sulfadiazine treatment. After acute i.v. treatment with atovaquone nanosuspension, mice were treated with oral maintenance regimens as indicated (see Materials and Methods and Results).
[b] *, $P < 0.0001$ compared to control mice; †, $P < 0.001$ compared to control mice; ‡, not significant compared to control mice.

DISCUSSION

Results of the present study reveal that mice treated orally with atovaquone maintenance therapy in doses equivalent to those administered in humans did not develop reactivation of TE.

These results were obtained in a new murine model of reactivated toxoplasmosis that closely mimics signs of reactivated toxoplasmosis in immunocompromised patients, including the presence of parasite-associated focal necrotic lesions in the brain parenchyma and meningeal inflammation. These pathological changes resulted in the death of untreated mice.

Studies on the efficacy of antiparasitic drugs for maintenance therapy have previously been performed by using in vitro systems and/or different animal models (1, 3, 4, 9, 24). However, none of these model systems represented reactivation of latent toxoplasmosis, and antiparasitic drugs for maintenance therapy were never administered orally after a course of acute therapy. Thus, the murine model described here for the first time allowed us to adequately study the efficacy of antiparasitic drugs for maintenance therapy.

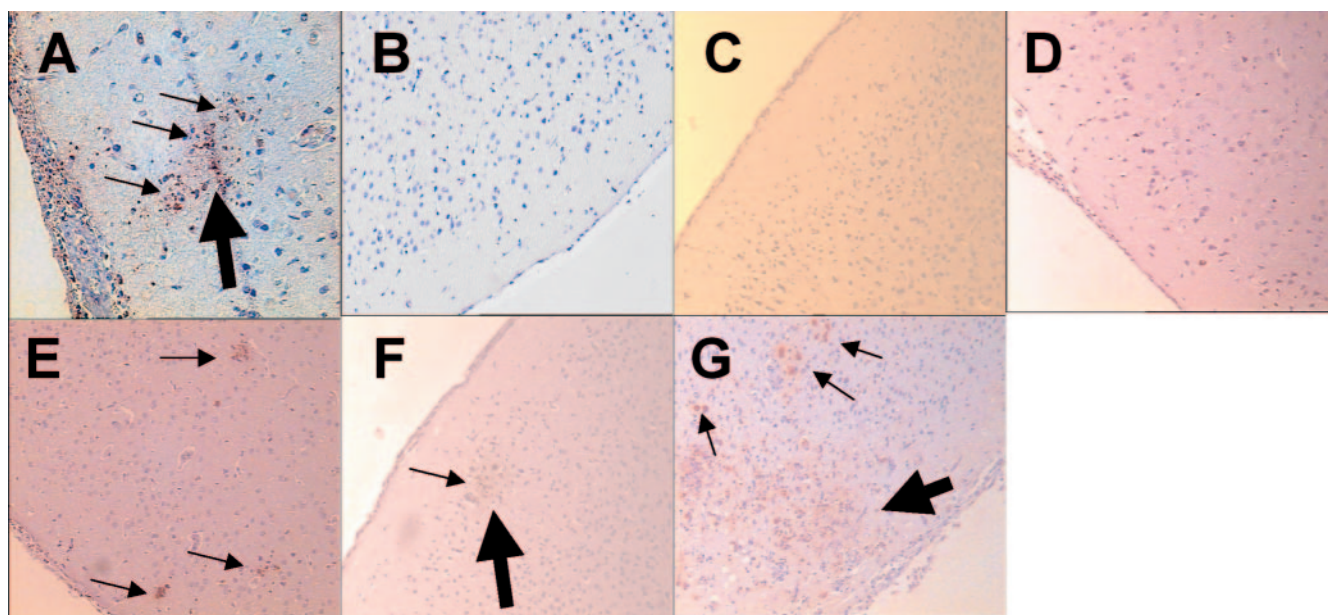The current recommendations for maintenance therapy

FIG. 3. Histologic changes in brains of mice with reactivated TE at day 8 after initiation of maintenance therapy. Small arrows indicate parasitic foci (parasitophorous vacuoles and parasitic antigen); large arrows indicate inflammatory foci. Immunoperoxidase staining was performed. Magnification, ×100. (A) Control mice; (B to G) mice treated with atovaquone at 100 mg/kg (B) or at 50 mg/kg (C), pyrimethamine plus sulfadiazine (D), pyrimethamine plus clindamycin (E), trovafloxacin (F), or sulfadiazine (G). The sections shown are representative for at least four mice per group; experiments were repeated three times.
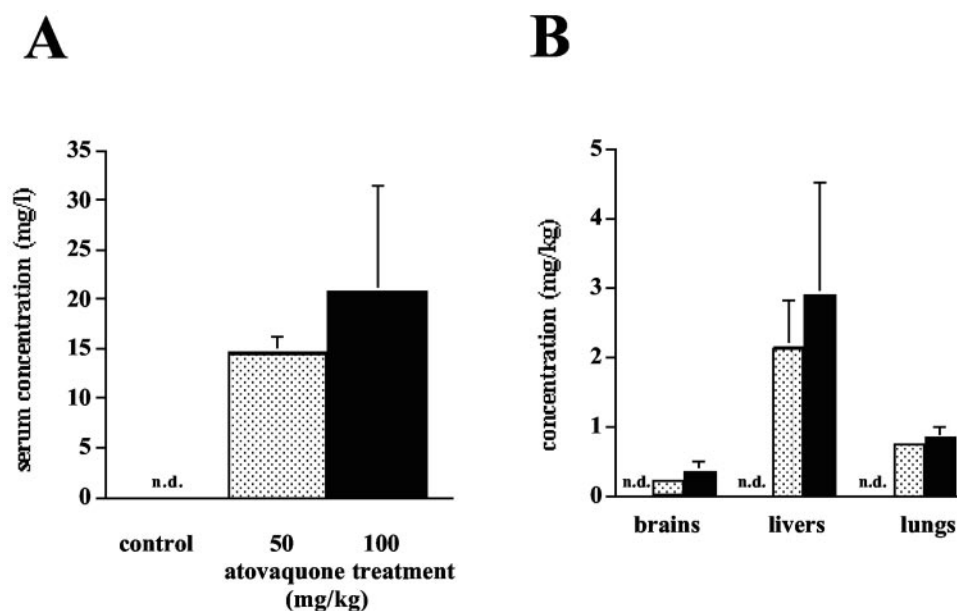
FIG. 4. Concentrations of atovaquone in sera (A) or brains, livers, and lungs (B) of mice with reactivated TE. Mice were treated with the indicated dosages of atovaquone (50 [strippled bars] or 100 [solid bars] mg/kg) and killed 7 days after initiation of oral atovaquone maintenance therapy (1 day after the last dose of atovaquone). Atovaquone concentrations were determined by MS (sera and brains) and HPLC (livers and lungs). Values are derived from at least three mice per group and are representative for two experiments.

against TE in patients with AIDS include as the first choice the use of sulfadiazine (500 to 1,000 mg orally four times daily) plus pyrimethamine (25 to 50 mg orally daily); this recommendation is based on the strong evidence for efficacy and clinical benefit observed in randomized clinical trials (A1 strength of recommendation by the U.S. Public Health Service and the Infectious Disease Society of America) (15). Alternatively, clindamycin (300 to 450 mg orally every 6 to 8 h) plus pyrimethamine (25 to 50 mg by mouth daily) or atovaquone (750 mg orally every 6 to 12 h) with or without pyrimethamine (25 to 50 mg orally daily) may be given; the evidence for the efficacy of these alternative treatments is based on clinical experience, descriptive studies, or reports of consulting committees (CIII strength of recommendation) and are therefore insufficient to support recommendation (15). In a study by Katlama et al. (17), 65 AIDS patients intolerant of standard treatment regimens received atovaquone (750 mg four times daily) as single maintenance therapy. Atovaquone was found to be efficacious in 74% of the patients (17). The efficacy of 74% observed in humans by Katlama et al. is lower than the efficacy of 100% observed in mice in the present study. However, whereas 750 mg of the old tablet form of atovaquone was administered four times daily to patients, mice received the new suspension formulation of atovaquone in equivocal doses. It has been shown that Hifat (toast with 56 g of butter) increased the uptake of atovaquone tablets by factors of 3.9 (area under the curve [AUC]) and 5.6 (C_{max}) (23). Atovaquone aqueous suspension or oily solution in miglyol also increased the AUC and C_{max} by factors of 1.7 and 2.4, respectively (23). We therefore hypothesize that the current formulation of atovaquone (oral suspension with xanthan gum and poloxamer 188 [Wellvone package insert 1997]) should prove markedly more efficacious than the old formulation for maintenance

therapy against reactivation of TE in humans. In this respect, the combination of atovaquone suspension plus either pyrimethamine or sulfadiazine was as effective as maintenance therapy in 19 (95%) of 20 patients with AIDS (5). However, the study by Chirgwin et al. (5) did not allow us to determine whether the new atovaquone formulation or rather the combination with another drug caused the protective effects.

In all regimens, including sulfadiazine or pyrimethamine, leucovorin therapy must be added to prevent bone marrow suppression. In addition, sulfadiazine and clindamycin therapy is hampered by allergic reactions, which are observed in as many as 20% of patients; between 11 and 30% of patients discontinue maintenance therapy (16). In contrast, atovaquone maintenance therapy has been reported to be very well tolerated (17). Only 2 (3.0%) of 65 AIDS patients experienced gastrointestinal side effects, including nausea and vomiting, and therefore had to discontinue atovaquone maintenance therapy (16).

We compared the therapeutic effect of oral administration of atovaquone in dosages between 25 and 100 mg/kg (body weight). Atovaquone dosages of 100 and 50 mg/kg were efficacious for maintenance therapy against TE, whereas atovaquone at a dose of 25 mg/kg did not protect mice from reactivated toxoplasmosis (data not shown). Dosages of 50 and 100 mg/kg resulted in levels of 14.5 and 20.8 mg/liter, respectively, in serum; levels achieved in the sera of infected mice thus fall above the MIC for *T. gondii* reported by us and others to be in the nanogram range (1, 3, 25) The dosage of 50 mg/kg that proved efficacious in the murine model of reactivated TE is equivalent to the dosage reported to be efficacious in humans (750 mg given four times daily) by Katlama et al. (17). Since we were interested in brain concentrations of atovaquone, an MS assay for the detection of atovaquone was established. Con-

centrations achieved in the brains of infected mice (0.22 to 0.34 µg/mg) also fell above the MIC of 0.01 µg/ml for *T. gondii*.

In the past, acute therapy for TE has been investigated in murine models by using drug-induced immunosuppression (10). In mice latently infected with the cystogenic ME49 strain, reactivation of TE was induced by a 2-week course of dexamethasone. Administration of atovaquone plus clindamycin (dosages of 50 mg/kg [body weight] each or higher) significantly prolonged the survival of mice and significantly reduced the numbers of brain cysts (10). A similar effect of atovaquone on cyst numbers was also reported by Araujo et al. (2) in vitro and in a murine model of chronic progressive toxoplasmosis in susceptible CBA/ca mice.

In conclusion, the murine model of reactivated toxoplasmosis described here proved valuable for studying the efficacy of antiparasitic drugs for maintenance therapy against TE. The therapeutic effect of atovaquone should be further evaluated in clinical trials.

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